REMARKS

Responsive to the preliminary determination of lack of unity set forth in the outstanding Official Action, applicants provisionally elect Group II, claims 15-17, 19, 23, 25, 31, and 32, directed to a method of transdifferentiating adipocytes into myoblasts, with traverse.

The reasons for traverse follow:

The position of the Official Action is that Groups I-V lack a common technical feature, as evidenced by PARK et al. ("PARK"). PARK is offered for allegedly teaching of transdifferentiating adipocytes into cells with an osteogenic phenotype exhibiting the markers of osteogenesis.

However, PARK fails to teach that for which it is offered.

Indeed, no one in the past has shown success in starting from a fat tissue sample. As discussed in the present specification, adult stem cells derived from bone marrow stromal have been used as donor cells in the past, but there are disadvantages to doing so.

PARK appreciated that the cells present in the floating low-density layer of human bone marrow are mainly adipocytes and preadipocytes. Single adipocytes are present together with adipocytes associated in conglomerates with fibroblastic cells. See, e.g., the discussion of the PARK experiments on page 553. The various stromal cells present in bone marrow tissue form

clusters with adipocytes, and these clusters are likely to exist in the "cells contained in a suspended fraction after centrifugation of the bone marrow samples", which is the "adipocyte" of PARK.

The prior art, e.g., JP 2000083656 of the IDS filed December 13, 2005, shows that in order to obtain a unilocular adipocyte, collagenase processing is needed, which includes multiple rounds of centrifugation and a filtration mesh of about 250μm.

PARK, however, fails to disclose or suggest any collagenase treatment and filtration. Moreover, PARK does not disclose checking the suspended fraction obtained to see if it is composed solely of adipocytes.

However, collagenase treatment and filtration are necessary, i.e., due to the tissue structure of bone marrow, to obtain a single fraction of adipocyte. Indeed, collagenase treatment and filtration are essential for the reasons discussed below:

The structure of bone marrow tissue includes reticular tissue that forms stromata and the hematopoietic cells fill the mesh of it. Reticular tissue consists of reticular cells and reticular fibers, thereby forming a microenvironment for induction of differentiation and modulation of hematopoietic cells. Reticular cells include fibroblast, preadipocyte, cells surrounding blood vessels, smooth muscle cells, bone marrow stern

cells, and so on, that produce reticular fibers (collagens). It is known that some of the reticular cells, preadipocytes, differentiate into adipocytes and fill the gap within the pith cavity when hematopoiesis is inactive. Thus, there must be stem cells which can differentiate into preadipocytes and adipocytes. These cells within the reticular tissue cannot be distinguished from one another because they all have similar shapes. Consequently, in order to isolate the adipocyte in the reticular tissue of bone marrow, it is necessary to fractionate the cells by digesting the reticular fibers with collagenase.

Collagenase is the enzyme used to digest the extracellular matrix which connects the various types of cells within adipose tissue. All tissue consists of a variety of different cell types but the digestive enzyme is required to digest the extracellular matrix to enable isolation of the cells from the tissue varies depending on the tissue. The isolation of the cells requires the follow up filtration step to remove undigested tissue. Centrifugation is used to force the adipocytes more to the upper layer fraction because lipid droplets abundant in the cytoplasm cause buoyancy while the other cells precipitate. Thus, the pure adipocyte can be isolated. Ceiling culture, as seen in the present application, enables floating adipocytes exclusively to be cultured. One skilled in the art at the time the invention was made was well aware that if the enzyme digestion and filtration process is not followed, the

adhesive cells recovered will contain various types of cells from the bone marrow tissue.

The obtaining of fibroblast-like adipocytes from the cell suspension containing only mature adipocytes is allegedly carried in PARK in sidewell plates. One skilled in the art would be aware that it is impossible for the suspended cells to attach to the bottom of the wells because they remain suspended within the upper portion of the culture and medium due to the large amount of lipid droplets in cytoplasm. In the present invention, the inventors performed ceiling culture to obtain the adipocytes. The adipocytes attach themselves to the surface of the dish allowing them to display proliferative capacity.

PARK, however, does not take this measure. Accordingly, the cell cluster containing adipocytes and bone marrow stromal cells, when cultured in six well culture dishes, is going to lead to fibroblasts falling off and there is doubt, therefore, as to the conclusions of PARK. These fibroblasts from bone marrow stromal cells could be ones which are subjected to later processing by PARK.

In order to obtain the fibroblast-like cells, PARK used a culture medium in which the fraction was induced to differentiate into an adipocyte. However, a culture medium for inducing differentiation usually has the potential of maintaining the function of the adipocyte and suppressing dedifferentiation. There is a contradiction here in using a culture medium to induce

differentiation in order to dedifferentiate the adipocyte. Yet it makes sense if the starting point is pluripotent stem cells, which will differentiate into adipocytes.

One skilled in the art would, therefore, doubt the disclosure of PARK as confirmation from bone marrow stromal cells.

There is no disclosure that the preadipocyte cell line in PARK expresses an early marker of osteogenesis, myogenesis or adipogenesis. One skilled in the art has read reports in a number of publications that, when using conventional preadipocyte strains, the early differentiation marker genes for adipogenesis are expressed only after induction of differentiation, but not before induction. One skilled in the art at the time was of the view the adipocyte maintained a function by expressing adipocyte specific genes. The absence of any mention of the early marker genes in PARK confirms that the identity of their cells remains unknown.

It is quite likely that the cells were originated from the bone marrow stromal cells, and if that is the case, it is not at all surprising that their adipocyte derived cells were induced to differentiate into adipocyte by the culture medium for induction of differentiation into adipocyte. Bone marrow stromal cell populations contain pluripotent stem cells.

This lack of clarity as to the cells actually used by PARK is such that PARK fails to teach the dedifferentiation of mature adipocytes derived from bone marrow followed by the transdifferentiation.

PARK tried to prove that the cells obtained were derived from adipocytes by using anti-AP2 antibody and Oil red O staining. In fact, differentiation to adipocyte can be confirmed by assessing the expression of ap2 gene that is known to be a late marker gene of the differentiation to adipocyte or by performing Oil red O staining of lipid adipocytes.

The cells obtained by PARK expressed those markers because they had been cultured in a culture medium which induces the differentiation to adipocyte. That is, these cells were the cells which had already been differentiated into adipocyte and possessed the function of adipocytes. In these differentiated cells, the expression of genes and proteins which are specifically expressed in other types of cells (bone, muscle and chondrocyte, for example) is suppressed. In order to differentiate into another cell type, the function of the adipocyte must be turned off by dedifferentiation. However, there appears to be no such measure taken for the cells obtained by PARK. The cells obtained must be different from the preadipocyte strain of the claimed invention. Indeed, the cells are most likely the cells derived from multilocular adipocytes

that have been induced by differentiation of bone marrow stromal cells that were contaminated during isolation.

Thus, for the reasons discussed above PARK cannot teach transdifferentiating adipocytes into cells with an osteogenic phenotype exhibiting the markers of osteogenesis, and there is no basis for a determination of lack of unity.

Therefore, withdrawal of the restriction requirement is respectfully requested.

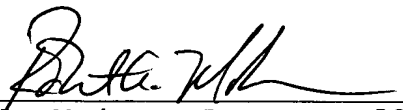
Furthermore, the Examiner's attention is respectfully directed to the paper included in the appendix, i.e., MATSUMOTO et al., "Mature Adipocyte-Derived Dedifferentiated Fat Cells Exhibit Multilineage Potential", which shows that the preadipocyte cell line of the present invention is a novel cell line which applicants have isolated from mature adipocytes.

A favorable action on the merits of all pending claims is respectfully requested.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

YOUNG & THOMPSON



Robert A. Madsen, Reg. No. 58,543
209 Madison Street
Suite 500
Alexandria, Virginia 22314
Telephone (703) 521-2297
Telefax (703) 685-0573
(703) 979-4709

RAM/snt

APPENDIX:

- MATSUMOTO et al., "Mature Adipocyte-Derived Dedifferentiated Fat Cells Exhibit Multilineage Potential", Journal of Cellular Physiology, 9999: 1-13, 2007.

Journal of Cellular Physiology

Mature Adipocyte-Derived Dedifferentiated Fat Cells Exhibit Multilineage Potential

TARO MATSUMOTO,[1] KOICHIRO KANO,[2] DAISUKE KONDO,[1] NOBORU FUKUDA,[3] YUJI IRIBE,[1] NOBUAKI TANAKA,[4] YOSHIYUKI MATSUBARA,[1] TAKAHIRO SAKUMA,[5] AYA SATOMI,[1] MUNENORI OTAKI,[4] JYUNNOSUKE RYU,[4] AND HIDEO MUGISHIMA[1,6]*

[1]*Division of Cell Regeneration and Transplantation, Advanced Medical Research Center, Nihon University School of Medicine, Tokyo, Japan*

[2]*Department of Animal Science, College of Bioresource Science, Nihon University, Fujisawa, Japan*

[3]*Advanced Research Institute of Science and Humanities, Nihon University, Tokyo, Japan*

[4]*Department of Orthopedic Surgery, Nihon University School of Medicine, Tokyo, Japan*

[5]*Department of Urology, Nihon University School of Medicine, Tokyo, Japan*

[6]*Department of Pediatrics, Nihon University School of Medicine, Tokyo, Japan*

When mature adipocytes are subjected to an in vitro dedifferentiation strategy referred to as ceiling culture, these mature adipocytes can revert to a more primitive phenotype and gain cell proliferative ability. We refer to these cells as dedifferentiated fat (DFAT) cells. In the present study, we examined the multilineage differentiation potential of DFAT cells. DFAT cells obtained from adipose tissues of 18 donors exhibited a fibroblast-like morphology and sustained high proliferative activity. Flow cytometric analysis revealed that DFAT cells comprised a highly homogeneous cell population compared with that of adipose-derived stem/stromal cells (ASCs), although the cell-surface antigen profile of DFAT cells was very similar to that of ASCs. DFAT cells lost expression of mature adipocytes marker genes but retained or gained expression of mesenchymal lineage-committed marker genes such as peroxisome proliferator-activated receptor gamma (PPARγ), RUNX2, and SOX9. In vitro differentiation analysis revealed that DFAT cells could differentiate into adipocytes, chondrocytes, and osteoblasts under appropriate culture conditions. DFAT cells also formed osteoid matrix when implanted subcutaneously into nude mice. In addition, clonally expanded porcine DFAT cells showed the ability to differentiate into multiple mesenchymal cell lineages. These results indicate that DFAT cells represent a type of multipotent progenitor cell. The accessibility and ease of culture of DFAT cells support their potential application for cell-based therapies.

J. Cell. Physiol. 9999: 1–13, 2007.

Mesenchymal stem cells (MSCs) are multipotent cells that were originally identified in adult bone marrow. MSCs can proliferate and differentiate into multiple mesodermal lineages (Pittenger et al., 1999). Recent studies have shown that adipose tissue represents an alternative source of MSCs (Zuk et al., 2001, 2002). Adipose tissue contains non-adipocyte cells known as the stromal-vascular fraction (SVF) that can be isolated by centrifugation of collagenase-digested adipose tissue. The cultured SVF has been termed adipose-derived stem/stromal cells (ASCs) (McIntosh et al., 2006; Mitchell et al., 2006). Both in vitro and in vivo studies have shown that human ASCs can differentiate along multiple lineages, including adipocytes, osteoblasts, chondrocytes, myocytes, neuronal cells, endothelial cells, and hepatocytes (Schaffler and Buchler, 2007). Compared to bone marrow MSCs, ASCs have potential advantages for tissue engineering application, because of the ease of isolation without painful procedures or donor site injury. However, like bone marrow MSCs, ASCs are a minor population of cells in the adipose tissue, and cultured ASCs are a heterogeneous cell population, especially at early passage numbers. Liposuction-derived ASCs at passage 0 contain contaminating endothelial cells, smooth muscle cells, and pericytes (Zuk et al., 2001). Expression of various endothelial cell markers by cultured ASCs does not change significantly with serial passage (McIntosh et al., 2006). Therefore, the other adult stem cell sources with high purity are still needed.

In contrast to ASCs, mature adipocytes are the most abundant cell type in adipose tissue. It has been shown that the mature adipocytes isolated from fat tissue can be dedifferentiated into fibroblast-like cells with an in vitro dedifferentiation strategy, known as ceiling culture. This method exploits the buoyant property of adipocyte, which allows them to adhere to the top inner surface of a culture flask

T. Matsumoto and K. Kano contributed equally to this work.

This article includes Supplementary Material available from the authors upon request or via the Internet at http://www.interscience.wiley.com/jpages/0021-9541/suppmat.

Contract grant sponsor: Ministry of Education, Science, Sports, and Culture of Japan;
Contract grant number: 1550863.
Contract grant sponsor: Nihon University Multidisciplinary Research Grant;
Contract grant number: 2005-2006.
Contract grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan.

*Correspondence to: Hideo Mugishima, Department of Pediatrics, Nihon University School of Medicine, 30-1, Ohyaguchi-Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan.
E-mail: mugishim@med.nihon-u.ac.jp

Received 17 July 2007; Accepted 27 August 2007

Published online in Wiley InterScience (www.interscience.wiley.com.), 00 Month 2007.
DOI: 10.1002/jcp.21304

that is filled completely with medium (Sugihara et al., 1986) or to the underside of a floating piece of glass (Zhang et al., 2000). The adipocyte-derived fibroblast-like cells show proliferative activity and can redifferentiate into mature adipocytes both in vitro and in vivo (Sugihara et al., 1986, 1987; Shigematsu et al., 1999; Yagi et al., 2004), indicating that these cells show characteristics of adipogenic progenitors. Our group has established an adipogenic progenitor cell line derived from mature adipocytes of ddY mice and named these cells dedifferentiated fat (DFAT) cells (Yagi et al., 2004). More recently, it was reported that mature adipocyte-derived dedifferentiated cells from ceiling cultures could differentiate into either osteoblasts (Justesen et al., 2004) or vascular endothelial cells (Planat-Benard et al., 2004). These findings prompted us to hypothesize that DFAT cells are multipotent cells like MSCs. In the present study, we show that DFAT cells can differentiate into adipogenic, osteogenic, and chondrogenic lineages under the proper culture conditions even though the cells are clonally expanded. In addition, flow cytometric analysis revealed that DFAT cells are a relatively homogeneous cell population compared with ASCs. Because adipose tissue is abundant and easily accessible at most ages, DFAT cells may be an attractive source of mesenchymal lineages for tissue engineering and other cell-based therapies.

Materials and Methods

Animals and tissue sampling

All animal experiments were performed in the laboratory according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The 5-week-old male BALB/c nu/nu nude mice and adult male pigs (body weight 35 kg) were purchased from CLEA Japan, Inc. (Tokyo, Japan). Samples of human subcutaneous fat were obtained from patients undergoing surgery in the Departments of Plastic Surgery, Urology, Pediatric Surgery and Orthopedic Surgery of Nihon University Itabashi Hospital (Tokyo, Japan). The patients gave written informed consent, and the Ethics Committee of Nihon University School of Medicine approved the study.

Cell isolation and culture

Isolation of mature adipocytes from fat tissue was done with a modification of the method described previously (Sugihara et al., 1986). Briefly, approximately 1 g of fat tissue was minced and digested in 0.1% (w/v) collagenase solution (Collagenase type I, Koken Co., Ltd., Tokyo, Japan) at 37°C for 1 h with gentle agitation. After filtration and centrifugation at 135*g* for 3 min, the floating top layer containing unilocular adipocytes was collected. After a wash with phosphate-buffered saline (PBS), cells (5×10^4) were placed in 25-cm^2 culture flasks (NUNC, Roskilde, Denmark) filled completely with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS, Lot 6G2146) and were incubated at 37°C in 5% CO_2. Cells floated up and adhered to the top inner ceiling surface of the flask. After 7 days, the medium was removed, and the flasks were inverted so that the cells were on the bottom. The medium was changed every 4 days until the cells reached to confluence. After splitting, the cells were used for experiments. To isolate ASCs, collagenase-digested adipose tissues were centrifuged at 135*g* for 3 min. The pellet was washed with PBS and resuspended in DMEM supplemented with 20% FBS. The cells (5×10^6) were incubated at 37°C in 5% CO_2 in 25-cm^2 culture flasks. The medium was changed every 4 days until the cells reached to confluence. After splitting, the cells were used for experiments. Human skin fibroblasts (CCD-27SK; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) were maintained in DMEM supplemented with 10% FBS.

Microscopy

Cells were fixed in 4% paraformaldehyde (Sigma–Aldrich, St. Louis, MO) for 15 min, washed once in PBS, and incubated 10 min with AdipoRed® (Cambrex, Walkersville, MD) to visualize lipid droplets. Cells were then washed in PBS and subjected to DNA staining with 5 μg/ml Hoechst 33342 (Sigma–Aldrich). Detection of S-phase cells was described previously (Matsumoto et al., 2002). Staining was visualized and photographed with an immunofluorescence microscope (Nikon Eclipse TE 2000-U; Nikon, Tokyo, Japan). To record adipocyte proliferation, adipocytes at 24 h after ceiling culture were incubated with 5 μg/ml Hoechst 33342 at 37°C for 40 min. Subsequently, time-lapse fluorescent images of adipocyte ceiling cultures were then taken every 15 min for 48 h with Lumina Vision software (version 2.0; Mitani Corporation, Tokyo, Japan).

Flow cytometric analysis

After treatment with 0.05% trypsin-EDTA (Invitrogen), human DFAT cells and human ASCs at passage 1 and human skin fibroblasts were suspended in PBS with 0.1% bovine serum albumin (Sigma–Aldrich) at a density of 5×10^5 cells per tube. After blocking, cells were stained for 30 min on ice with various anti-human mAb unconjugated or conjugated with FITC, phycoerythrin (PE), or allophycocyanin (APC). The following mAbs were used: anti-CD13, anti-CD29-APC, anti-CD31-PE, anti-CD34-PE, anti-CD44-FITC, anti-CD45-FITC, anti-CD49d-PE, anti-CD56-PE, anti-CD90, anti-CD105, anti-CD106-PE, anti-human leukocyte antigen (HLA)-A,B,C-FITC, anti-HLA-DR-PE, (all from BD Biosciences[Q2]); anti-CD-11b-FITC (Beckman Coulter, Fullerton, CA); and anti-αsmooth muscle actin (DakoCytomation, Glostrup, Denmark). Binding of unconjugated anti-CD13, anti-CD90, anti-CD105, and anti-αsmooth muscle actin was detected by secondary staining with PE-conjugated anti-mouse IgG antibody (BD Biosciences). Cells were analyzed with a FACSCalibur flow cytometer with the CellQuest software package (Becton Dickinson, Bedford, MA). Positive cells were counted and compared with signal of corresponding immunoglobulin isotypes.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells with an Isogen RNA Extraction Kit (Nippon Gene Co., Tokyo, Japan). Total RNA (1 μg) was reverse transcribed into cDNA with random 9-mers with a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio, Ohtsu, Japan). The primers and the probes for Runx2 (Hs00231692_m1), osteopontin (Hs00167093_m1), osterix (Hs00541729_m1), lipoprotein lipase (LPL; Hs00173425_m1), leptin (Hs00174877_m1), glucose transporter 4 (GLUT4; Hs00168966_m1), peroxisome proliferator-activated receptor gamma (PPARγ; Hs00234592_m1), CAAT/enhancer-binding protein (C/EBP)α, (Hs00269972_s1), C/EBPβ (Hs00270923_s1), C/EBPδ (Hs00270931_s1), SOX9 (Hs00165814_m1), fibroblast growth factor (FGF)10 (Hs00610298_m1), perlecan (Hs00194179_m1), and aggrecan (Hs00153936_m1) were TaqMan Pre-Developed Assay Reagents (Applied Biosystems, Foster City, CA). Pig sequence-specific primers for SYBR Green assay were designed with Primer Express 1.0 software (Applied Biosystems). The primers for each gene are listed in the Supplementary Table. 18S ribosomal RNA for human (HS99999901_S1, Applied Biosystems) and pig (QuantumRNA 18S, no. 1716, Ambion, Austin, TX) were included as endogenous normalization controls to adjust for unequal amounts of RNA. mRNA was quantified with an ABI Prism 7300 (Applied Biosystems). Each sample (each reaction, 5 μl cDNA; total volume, 25 μl) was run in triplicate. Cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative gene expression was analyzed with the comparative Ct method with 18S ribosomal RNA as the endogenous control after confirming that the efficiencies of the

target and the endogenous control amplifications were approximately equal. Results are presented as target gene expression normalize to that of 18S ribosomal RNA.

Differentiation assays

For adipogenic differentiation, DFAT cells were plated in 30-mm dishes (BD Falcon, Franklin Lakes, NJ) at a density of 5×10^4 cells and grown to confluency. Cells were incubated for 3 weeks in DMEM containing 10% FBS, 1 μM dexamethasone (Sigma–Aldrich), 0.5 mM isobutylmethylxanthine (Sigma–Aldrich), 1 × insulin-transferrin-selenium-X (ITS; Invitrogen). Differentiated cells were fixed for 1 h with 4% paraformaldehyde, incubated in 50% ethanol for 3 min, and stained with Oil red O (Sigma–Aldrich) for 15 min. At the indicated time points, total RNA was extracted and subjected to real-time RT-PCR analysis.

For osteogenic differentiation, cells were grown to confluency in 30-mm dishes and incubated for 3 weeks in DMEM containing 10% FBS, 100 nM dexamethasone, 10 mM β-glycerophosphate (Sigma–Aldrich), and 50 μM L-ascorbic acid-2-phosphate (Sigma–Aldrich). Induction medium was replaced every 3 days. At the indicated time points, differentiated cells were fixed for 1 h with 4% paraformaldehyde and rinsed with PBS. For the detection of alkaline phosphatase activity (ALP), cells were incubated at 37°C for 1 h with 0.16% naphthol AS-TR phosphate (Sigma–Aldrich) and 0.8% Fast Blue BB (Wako, Osaka, Japan) dissolved in 0.1 M Tris buffer (pH 9.0). For the detection of calcium deposition, cells were incubated in 1% alizarin red S (Sigma–Aldrich) for 3 min. At the indicated time points, total RNA was extracted and subjected to real-time RT-PCR analysis. Soluble osteocalcin levels in cultures were detected with an enzyme-linked immunosorbent assay (ELISA, Takara Bio).

For chondrogenic differentiation, DFAT cells were grown to confluency in 30-mm dishes and incubated for 3 weeks in DMEM containing 1% FBS, 50 μM L-ascorbic acid-2-phosphate, 40 μg/ml proline (Sigma–Aldrich), 100 μg/ml pyruvate (Sigma–Aldrich), 10 ng/ml transforming growth factor (TGF)-β3 (R&D Systems, Minneapolis, MN), and 1 × ITS. Induction medium was replaced every 3 days. At the indicated time points, differentiated cells were fixed for 1 h with 4% paraformaldehyde and rinsed with PBS. Accumulation of chondrocyte matrix was detected with alcian blue staining (pH 2.5, Wako). For immunohistochemical staining, differentiated DFAT cells were incubated with anti-human collagen type II mAb (1:200 dilution, Daiichi Fine Chemical, Toyama, Japan) or anti-human aggrecan mAb (1:100, Chemicon, Temecula, CA) followed by incubation with chick anti-mouse IgG-Alexa-594 (1:1,000, Invitrogen). At the indicated time points, total RNA was extracted and subjected to real-time RT-PCR analysis. For pellet culture, cells were seeded at a density of 2×10^5 cells per pellet in 15-cm^3 conical tubes. Cells were gently centrifuged at 150g for 5 min to the bottom of the tubes and allowed to form compact cell pellets at 37°C under 5% CO_2. Cells were maintained as a small pellet in the chondrogenic differentiation medium for 3 weeks. The culture medium was replaced twice each week. The pellet was fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections of the pellet were cut at a thickness of 5 μm and stained with hematoxylin-eosin (HE).

In vivo bone formation

Human DFAT cells were seeded on 10 mm × 10 mm × 5 mm β-tricalcium phosphate (TCP)/collagen I sponges (Collagraft®, NeuColl, Campbell, CA) at a concentration of 3×10^6 cell/matrix and were incubated in osteogenic medium for 2 weeks. The sponges with cells were implanted subcutaneously into nude mice (male, 6-week old). After 3 weeks, the animals were killed, and the implants were removed and photographed. The implants were then decalcified in HCl, processed, embedded in paraffin, and sectioned (6 μm). Masson Trichrome staining was then performed.

Clonal cell culture

To obtain clonal DFAT cells, single porcine DFAT cells were picked up with a micropipet under direct microscopic visualization and cultured in proliferation medium composed of 50% fresh, 20% FBS/DMEM, and 50% conditioned medium (prepared from human DFAT cell culture) supplemented with 20 ng/ml FGF2 (Peprotech, Rocky Hill, NJ) in each well of a 96-well plate (Corning, Inc., Corning, NY) for 14 days. Each clone was dissociated and transferred to a single well of a 24-well plate (BD Falcon), and after 7–10 days in proliferation medium, cells were again dissociated and transferred to six-well plates (BD Falcon). After an additional 1 week of culture in proliferation medium, cells were transferred to a 75-cm^2 culture flask (Corning, Inc.) in a final volume of 10 ml proliferation medium. Clonal lines were then maintained as regular passaged DFAT cells and used for differentiation studies before they reached passage 5.

Results

Establishment of DFAT cells from adipose tissue

The ceiling culture method is illustrated in Figure 1. Human subcutaneous adipose tissue was obtained from a total of 18 donors [7 males and 11 females; age, 52.7 ± 27.9 years (mean ± SD); range, 1–81 years] who underwent surgery. Adipose tissue samples were digested with collagenase followed by filtration and centrifugation. The floating top layer containing mature adipocytes was collected. Microscopic analysis of the isolated cells stained for neutral lipid by AdipoRed® and for nuclei by Hoechst 33342 revealed that 97.2 ± 1.2% of the cells were lipid-filled mature adipocytes with a single nucleus (Fig. 2A). When isolated adipocytes were cultured in flasks filled completely with medium, the cells floating on top of the medium attached to the upper surface of the flasks within a few days. Approximately 40% of the adherent cells flattened out by day 3 (Fig. 2B) and showed cell motility (Supplementary video). In the BrdU incorporation assay, approximately 50% of the adherent cells were positive for BrdU on day 3 (Fig. 2C), whereas the isolated mature adipocytes we examined were all negative for BrdU. This finding indicates that DNA synthesis in mature adipocytes was induced by the ceiling culture method. Time-lapse video images of cultured adipocytes prelabeled with Hoechst 33342 clearly showed that the adipocytes with a single nucleus divided asymmetrically and produced fibroblast-like cells (Fig. 2D,a–f and Supplementary video). Some adipocytes initially changed their morphology to a fibroblast-like morphology by releasing lipid droplets. These fibroblast-like cells then divided symmetrically and formed a colony by day 7. At this stage, most colony-forming cells contained small lipid droplets that were positive for AdipoRed®, indicating that the cells originated from mature adipocytes (Fig. 1E). Cells entered a proliferative log phase when the culture medium was changed and the flasks were inverted on day 7 of the ceiling culture. During this stage, the cells lost the lipid droplets completely and exhibited a spindle-shaped morphology (Fig. 1F). We refer to these cells as DFAT cells in this paper. As many as 3×10^7 DFAT cells were generated at the primary culture from 5×10^4 of mature adipocytes isolated from less than 1 g of human subcutaneous adipose tissue. The doubling time of DFAT cells was 65 h at passage 2 and 48 h at passage 10 (Fig. 1G). DFAT cells maintained their proliferative potential even at passage 11 with low frequency (<6%) of cellular senescence as detected by senescence-associated β-galactosidase staining (data not shown). DFAT cells were obtained from all tissue samples tested, although reduced proliferative activity was observed in cells from donors over 70 years of age. These results indicate that mature adipocytes can switch phenotype to fibroblast-like DFAT cells, which posses cell proliferative ability, in response

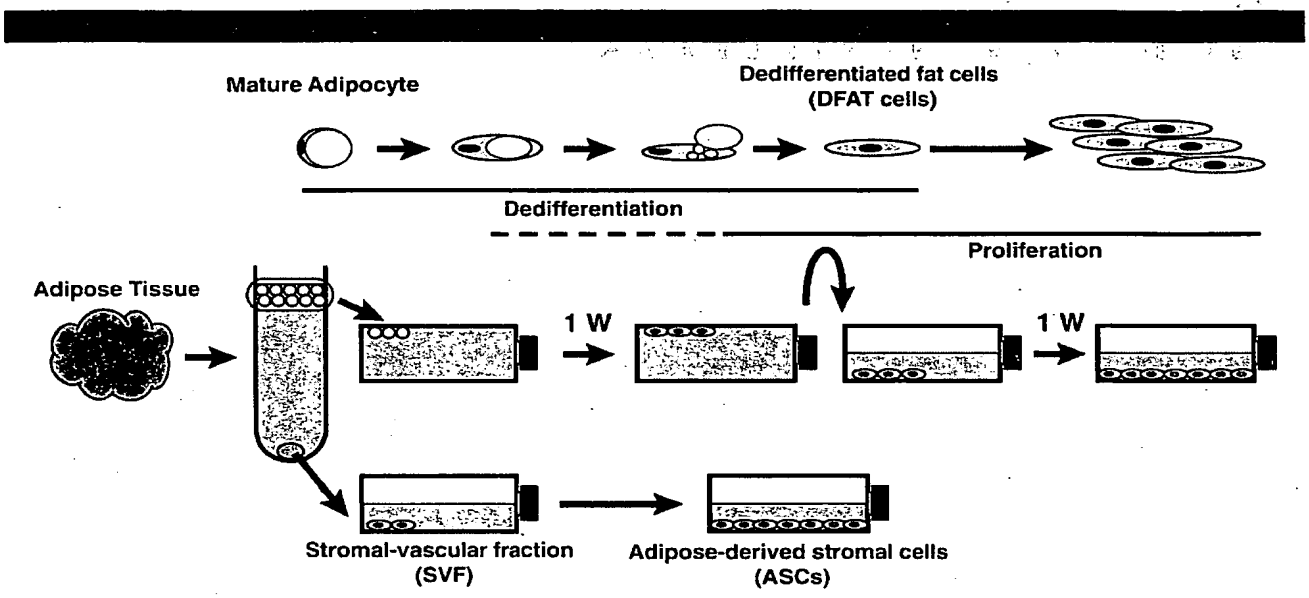


Fig. 1. Isolation, dedifferentiation, and culture of mature adipocytes. A small piece of adipose tissue was digested with 0.1% collagenase. After centrifugation, unilocular adipocytes were isolated from the floating layer. The isolated cells were cultured in culture flasks filled completely with DMEM containing 20% FBS for 7 days. During culture, the cells attached and flattened to the upper surface of the flasks followed by conversion to fibroblast-like dedifferentiated fat (DFAT) cells. Then the flasks were inverted and DFAT cells were cultured under conventional methods. Adipose-derived stem/stromal cells (ASCs) were obtained by expansion of adherent cells derived from pellets of collagenase-digested adipose tissue.

to ceiling culture. ASCs also could be isolated and expanded from cell pellets of collagenase-digested adipose tissue in consist with previous reports (Strem et al., 2005).

Expression of cell-surface antigens by DFAT cells

The cell-surface antigen profile of human DFAT cells at passage 1 was analyzed and compared with profiles of ASCs and primary cultures of human skin fibroblasts. DFAT cells were uniformly positive for CD13 (aminopeptidase N), CD29 (integrin β1), CD44 (hyaluronate receptor, phagocytic glycoprotein-1), CD49d (integrin α4-subunit), CD90 (Thy-1), CD105 (endoglin), and HLA-A, -B, and-C, but negative for CD11b, CD31 (platelet endothelial cell adhesion molecule), CD34, CD45, CD106 (vascular cell adhesion molecule-1), and HLA-DR (Fig. 3A,B). This profile is consistent with previous findings for bone marrow MSCs (Pittenger et al., 1999; Pittenger and Martin, 2004) except for CD49d and CD106 (Table 1). The cell-surface antigen profile of ASCs at passage 1 was essentially the same as that of DFAT cells. It has been reported that ASCs are a heterogeneous population that includes smooth muscle cells, endothelial cells, mast cells, and lineage-committed progenitor cells (Pettersson et al., 1985; Zuk et al., 2001; Yoshimura et al., 2006). Indeed, our flow cytometric analysis revealed that ASCs contained 18.6% α-smooth muscle actin-positive cells, 12.8% CD-45-positive cells, 13.3% CD11b-positive cells, and 2.7% CD31-positive cells, indicating contamination by smooth muscle cells, lymphocytes, monocytes, and endothelial cells, respectively (Fig. 3B). In contrast, DFAT cells contained almost no cells (0.00–0.07%) expressing these markers. These findings suggest that DFAT cells are a much more homogeneous population of mesenchymal cells than are ASCs. Human skin fibroblasts showed a profile similar to that of DFAT cells and ASCs, but the expression of CD105 and CD49d tended to be weaker, and CD56 (neural cell adhesion molecule) was strongly positive in fibroblasts (Table 1).

To examine changes in the gene expression profile during dedifferentiation culture, we used real-time RT-PCR to analyze the expression of several cell lineage-specific markers in DFAT cells and compared the profile with that of lipid filled mature adipocytes. Real-time RT-PCR detected abundant expression of mature adipocyte markers including LPL, leptin, and GLUT4 in mature adipocytes, whereas these markers were not expressed in DFAT cells (Fig. 4). In addition, the expression of transcription factors that are known to be involved in the regulation of adipocytic differentiation, such as PPARγ, C/EBPα, C/EBPβ, and C/EBPδ, was significantly down-regulated during the dedifferentiation culture, although these genes with the exception of C/EBPα, were still expressed. DFAT cells also expressed Runx2 and SOX9, critical transcription factors for osteogenesis and chondrogenesis, respectively. These results suggest that DFAT cells lose the functional characteristics of mature adipocytes but retain or gain the characteristics of adipogenic-, osteogenic-, and chondrogenic-lineage committed cells.

DFAT cells exhibit multilineage differentiation potential

The differentiation potential of DFAT cells was analyzed by culturing the cells under conditions that were favorable for adipogenic, osteogenic, or chondrogenic differentiation. We and other groups have reported that DFAT cells could redifferentiate into adipocytes (Sugihara et al., 1986; Zhang et al., 2000; Yagi et al., 2004). Consistent with previous observations, accumulation of lipid-rich vacuoles within cells was observed after adipogenic induction for 3 weeks (Fig. 5A). Upon adipogenic induction, real-time RT-PCR analysis revealed increased expression of functional adipocyte markers, including

COLOR

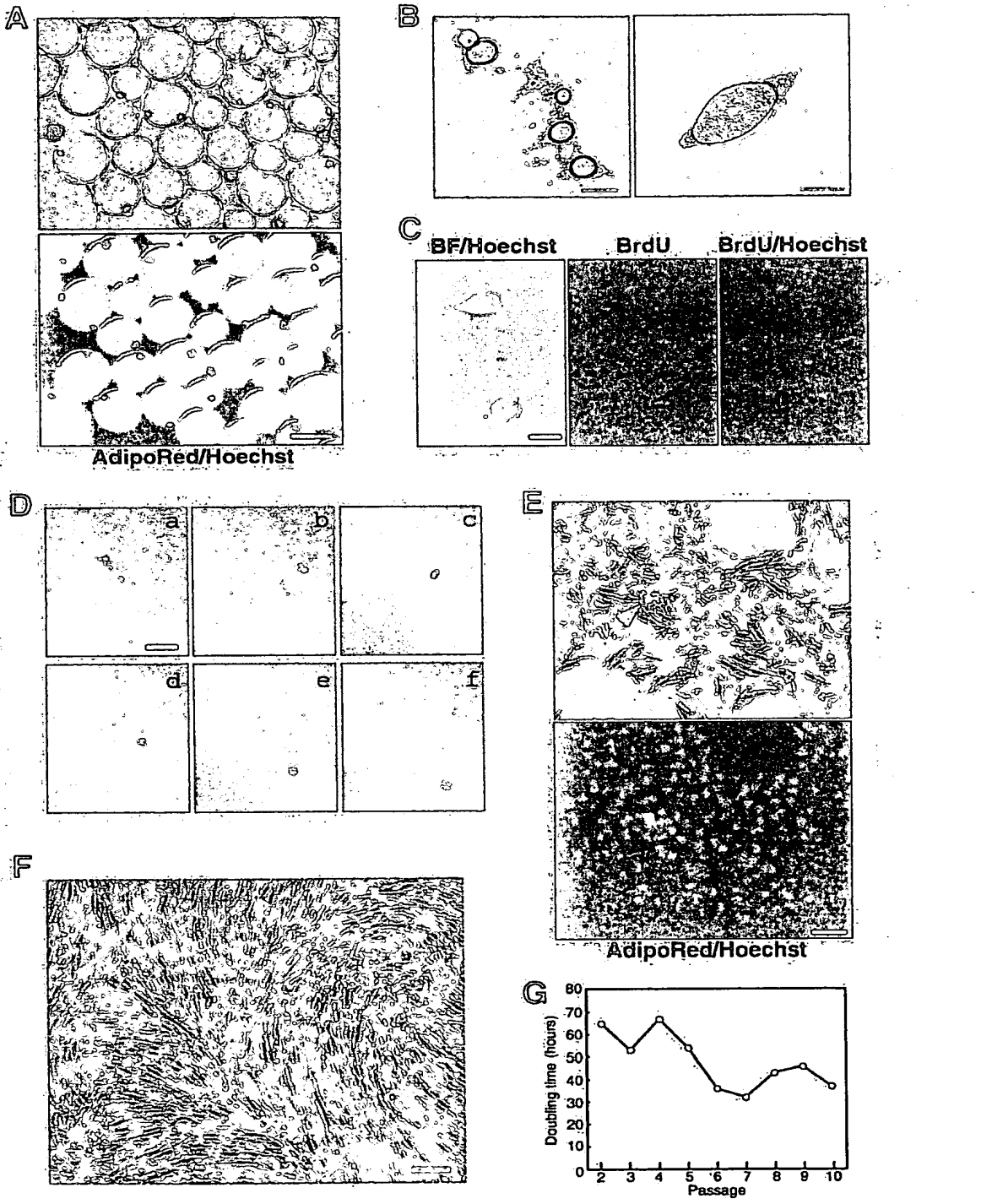


Fig. 2. Morphological changes from mature adipocytes to DFAT cells by ceiling culture method. A: Morphology of isolated mature adipocytes. Bar, 100 μm. B,C: Morphology of adipocytes on day 3 of culture. B: Phase-contrast microscopy showed that the cells extend cytoplasm. Bar in left part, 50 μm; right part, 25 μm. C: Incorporation of BrdU was frequently detected in adherent cells (upper cell). Bar, 25 μm. D: Time-lapse fluorescence microscopy revealed that the cell with a single nucleus divided asymmetrically and generated fibroblast-like cells (a–f). Bar, 50 μm. E: Morphology of adipocytes on day 7 of culture. The DFAT cells derived from an unilocular adipocyte (arrowhead) proliferated and formed a colony. Bar, 100 μm. F: Cells rapidly expanded and reached confluence on day 14 of culture. Bar, 200 μm. G: Growth kinetics of DFAT cells from passages 2 to 10.

COLOR

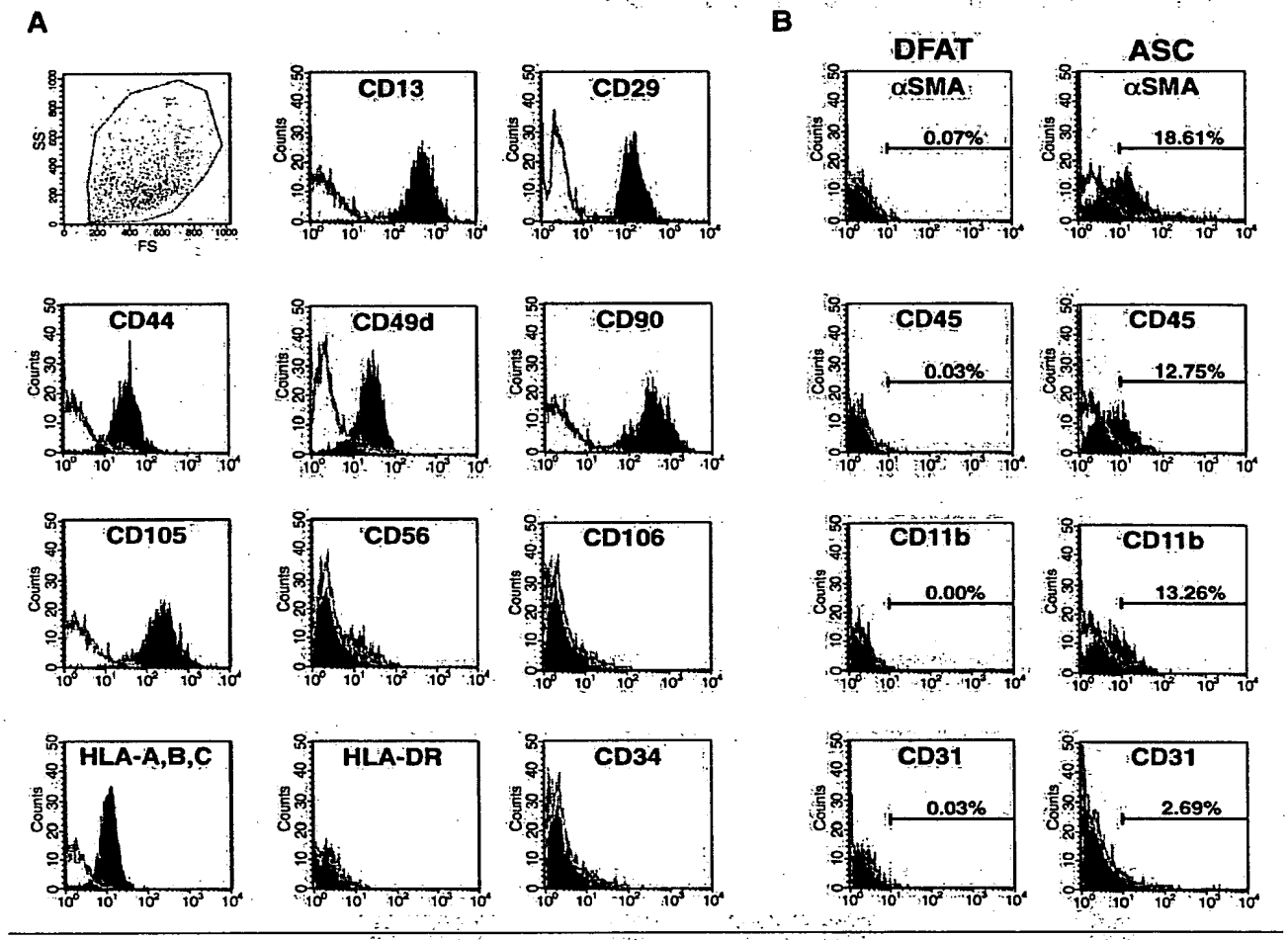


Fig. 3. Phenotypic analysis of human DFAT cells. A: Expression of cell-surface antigens on human DFAT cells harvested at passage 1. Plots show isotype control immunoglobulin G staining profile (green open histograms) versus specific antibody staining profile (blue closed histograms). B: Expression of α-smooth muscle actin (αSMA), CD45, CD11b, and CD31 on human DFAT cells and ASCs harvested at passage 1. Data are representative of three experiments.

LPL, leptin, and GLUT4, as well as several adipogenic differentiation markers, including PPARγ, C/EBPα, C/EBPβ, C/EBPδ, and FGF10 (Yamasaki et al., 1999) (Fig. 5B). In contrast, adipogenic differentiation was not induced in human skin fibroblasts cultured in the adipogenic induction medium (data not shown).

TABLE 1. Expression pattern of CD antigens in DFAT cells and the other type of cells

CD number		DFAT cells	BM MSCs	ASCs	Fibroblasts
CD13	Aminopeptidase[Q3] N	+	+	+	+
CD29	Integrin β1	+	+	+	+
CD44	Pgp-1	+	+	+	+
CD90	Thy-1	+	+	+	+
CD105	Endoglin	+	+	+	+Low
CD49d	Integrin α4	+	−	+	+Low
CD106	VCAM-1	−	+	−	−
CD56	NCAM isoform	−	−	−	+
CD34	L-selectin ligand	−	−	−	−
CD31	PECAM-1	−	−	−	−
CD11b	Mac-1	−	−	±	±
CD45	LCA	−	−	±	±

DFAT, dedifferentiated fat; BM MSCs, bone marrow mesenchymal stem cells; ASCs, adipose-derived stem/stromal cells; Pgp-1, phagocytic glycoprotein-1; VCAM, vascular cell adhesion molecule; NCAM, neuronal cell adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; LCA, leukocyte common antigen.

When human DFAT cells were cultured in the osteogenic induction medium, the cells maintained their fibroblast-like morphology and expressed alkaline phosphatase, a characteristic of osteoblasts, from 1 week of culture (Fig. 6A). Mineralized matrix aggregates were observed after 3 weeks of culture by staining for alizarin red S. Cultured skin fibroblasts did not exhibit ALP when cultured for as long as 4 weeks in the osteogenic induction medium. Real-time RT-PCR analysis revealed increased expression of Runx2, osteopontin, and osterix in DFAT cells during induction culture (Fig. 6B). Expression of osteocalcin, an osteoblast-associated matrix protein, has been considered a valid marker for fully differentiated osteoblasts. Protein levels of osteocalcin were significantly increased in DFAT cells after 3 weeks of induction culture (Fig. 6C). These results indicate that human DFAT cells can differentiate into osteoblasts in vitro.

We next examined whether the human DFAT cells could form osteoid tissue in vivo. Human DFAT cells (3×10^6) were loaded onto β-TCP/collagen I sponges (Collagraft®), and cultured for 2 weeks in osteogenic induction medium, followed by subcutaneous implantation into nude mice. After 3 weeks,

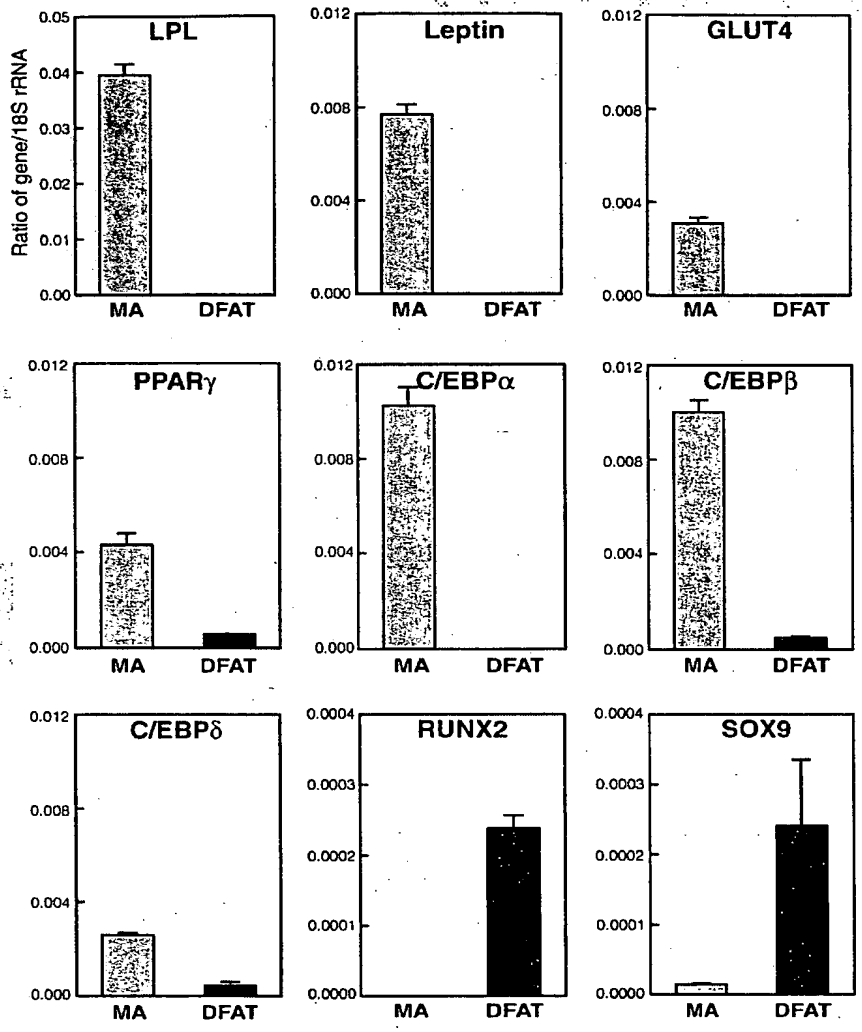


Fig. 4. Real-time RT-PCR analysis of mature adipocytes and DFAT cells. RT was carried out for RNA samples extracted from human mature adipocytes (MA) and from human DFAT cells (DFAT). Quantitative real-time PCR was performed with primers specific for LPL, Leptin, GLUT4, PPARγ, C/EBPα, C/EBPβ, C/EBPδ, Runx2, and Sox9. Data were analyzed with the comparative Ct method as described in the Materials and Methods Section. Values are mean ± SD of triplicate dishes.

implants were removed and evaluated morphologically and histologically for evidence of bone formation. Removed implants showed solidified bone tissues with vascular invasion (Fig. 6D, left part). The implants produced a mineralized matrix as shown by alizarin red S staining (Fig. 6D, right part). Histological analysis revealed that the implants contained osteoid, woven bone, osteoblast-like cells, and osteoclast-like cells (Fig. 6E). Osteoid tissue formation was not observed after transplantation of β-TCP/collagen I sponges alone or sponges with human fibroblasts. These results indicate that DFAT cells can form osteoid matrix in vivo.

When human DFAT cells were cultured in the chondrogenic induction medium, the DFAT cells formed micromasses in the culture and showed positive immunostaining for collagen type II, a marker of chondrocytes (Fig. 7A). The micromasses were also positive for alcian blue staining (Fig. 7B), suggesting the accumulation of cartilaginous proteoglycans. Pellet culture of DFAT cells for 3 weeks resulted in extensive accumulation of extracellular matrix and cell lacunae in a morphological pattern consistent with immature cartilage tissue (Fig. 7C). The tissue contained a multicellular peripheral layer of flattened, elongated, fibroblast-like cells similar to perichondrium. Real-time RT-PCR analysis showed increased expression of SOX9, an early differentiation marker for chondrocytes, and perlecan and aggrecan, markers of mature chondrocytes, after chondrogenic induction (Fig. 7D). These results indicate that human DFAT cells can undergo chondrogenic differentiation.

Clonal analysis of DFAT cells

We next used the in vitro differentiation assay with expanded colonies of cells derived from a single DFAT cell to exclude the possibility that the cell populations are composed only of committed progenitor cells. In this experiment, we first tried to expand human DFAT cell clones but failed to generate sufficient numbers of cells due to weak expansion from single cells.

COLOR

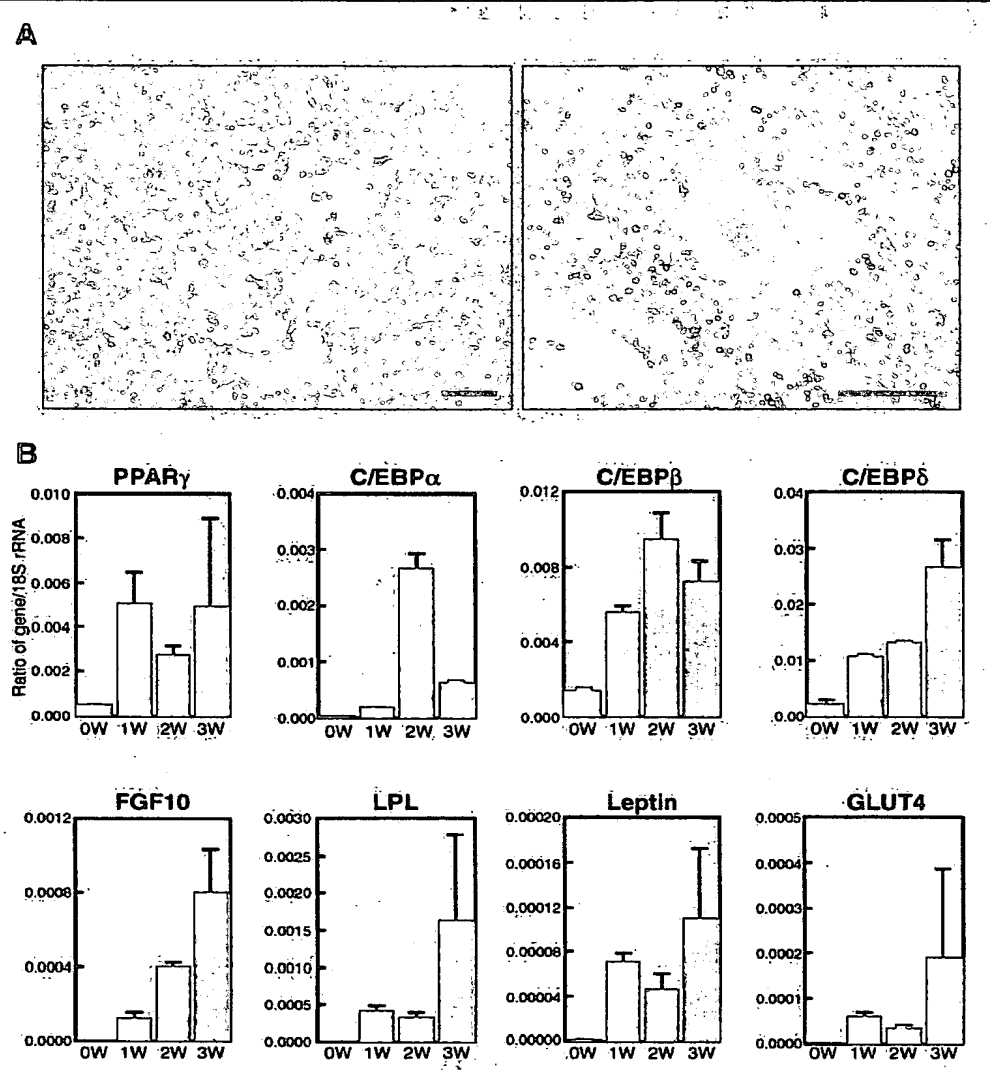
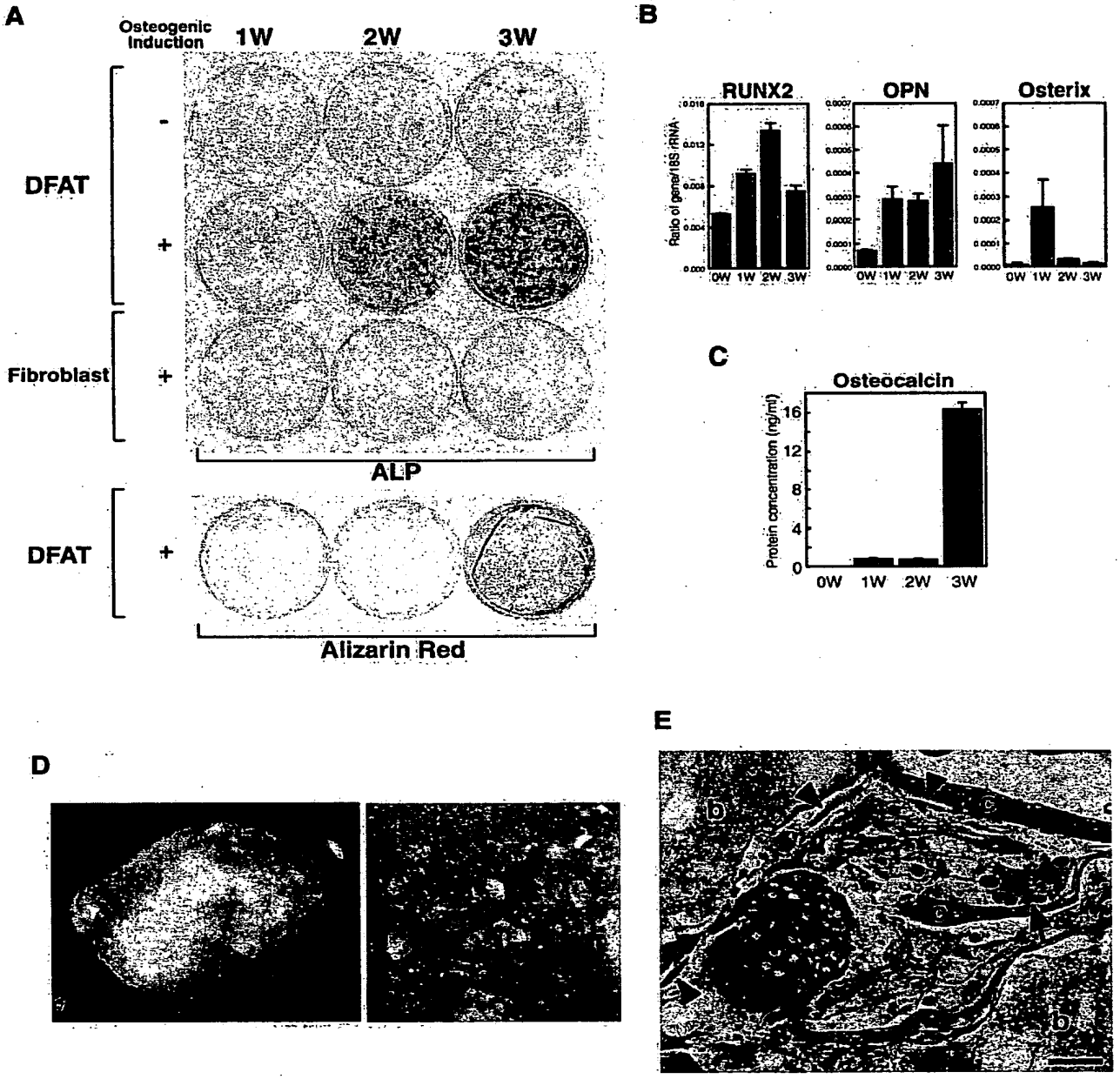


Fig. 5. Adipogenic differentiation of DFAT cells. A: Confluent human DFAT cells were cultured for 3 weeks in adipogenic medium. Cells were stained positively for lipid vacuole with Oil red-O, indicating adipogenic differentiation. Bar, 100 μm in left part and 50 μm in right part. B: Expression of adipogenic genes in DFAT cells was analyzed by real-time RT-PCR. Values are mean ± SD of triplicate dishes.

Therefore, we used DFAT cells derived from porcine subcutaneous adipose tissue because these cells show strong expansion ability even from single cells. Of more than 100 DFAT cells individually plated in 96-well plates, 13 clones were able to reach confluence. Eight of these clones were obtained after three passages without evidence of senescence and were used for differentiation analysis. Of the eight clones, two were able to differentiate into all three induced lineages (osteogenic, adipogenic, and chondrogenic differentiation, Fig. 8A), three were able to differentiate into two lineages (two osteogenic and adipogenic, one osteogenic and chondrogenic), and three were able to differentiate into only one lineage (two adipogenic, one osteogenic). The multipotent DFAT clone showed increased expression of adipogenic, osteogenic, and chondrogenic marker genes when cultured in adipogenic, osteogenic, and chondrogenic induction media, respectively (Fig. 8B). These findings indicate that DFAT cells have a multipotent capacity for lineage differentiation and are not solely a mixed population of unipotent progenitor cells.

Discussion

In the present study, we showed that mature adipocyte-derived DFAT cells enter the cell cycle, lose mature adipocyte markers, and have the ability to differentiate into multiple mesenchymal cell lineages. Urodele amphibians, such as newts, are known to regenerate amputated limbs and tails by cellular dedifferentiation (Tsonis, 2000; Brockes and Kumar, 2002; Del Rio-Tsonis and Tsonis, 2003). In mammals, terminally differentiated cells are normally incapable of reversing the differentiation process (Andres and Walsh, 1996; Walsh and Perlman, 1997). However, recent studies have provided evidence that terminally differentiated mammalian cells can dedifferentiate when the cells are cultured under special



COLOR

Fig. 6. Osteogenic differentiation of DFAT cells. A: Confluent human DFAT cells and human skin fibroblasts were cultured for 1–3 weeks in osteogenic medium or in growth medium. Cells were stained for alkaline phosphatase activity (ALP) and for alizarin red S to visualized calcified extracellular matrix. B: Expression of osteoblast marker genes in DFAT cells was analyzed by real-time RT-PCR before (0 W) and after osteogenic differentiation culture (1–3 W). OPN, osteopontin. C: Protein levels of osteocalcin were measured in DFAT cells by ELISA. D,E: Tissue-engineered bone generated by β-TCP/collagen matrix implant seeded with DFAT cells. Human DFAT cells were seeded on β-TCP/collagen matrices (Collagraft®) and cultured in osteogenic medium for 2 weeks. The cell/matrix composites were then implanted subcutaneously into nude mice. Three weeks later, the implants were removed and photographed (D). Demineralized samples were stained with Masson Trichrome (E). Osteoblast-like cells (arrowheads), osteoclast-like cells (arrow), mineralizing osteoid tissue (red), and woven bone (blue) were observed in the sample. b and c indicate β-TCP matrix and collagen fibers, respectively. Bar, 50 μm.

conditions. Mouse C2C12 myotubes can dedifferentiate and enter the cell cycle when stimulated with an extract prepared from newt regenerating limb tissue (McGann et al., 2001). Treatment with reversine induced dedifferentiation of muscle-committed C2C12 cells into multipotent progenitor cells (Chen et al., 2004). The results of the present study and previous studies (Sugihara et al., 1986, 1987, 1989; Zhang et al., 2000) suggest that mammalian mature adipocytes are an alternative cell type that can dedifferentiate ex vivo. Indeed, our present data revealed that mature adipocytes enter the cell

COLOR

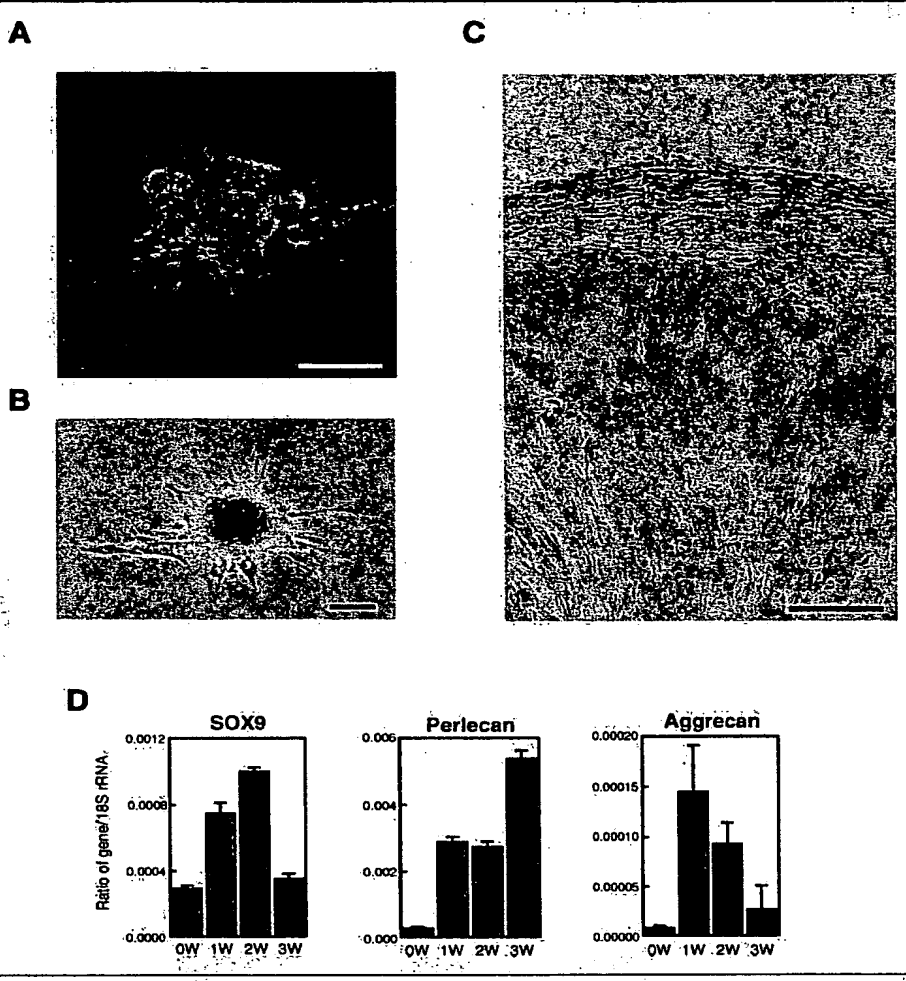
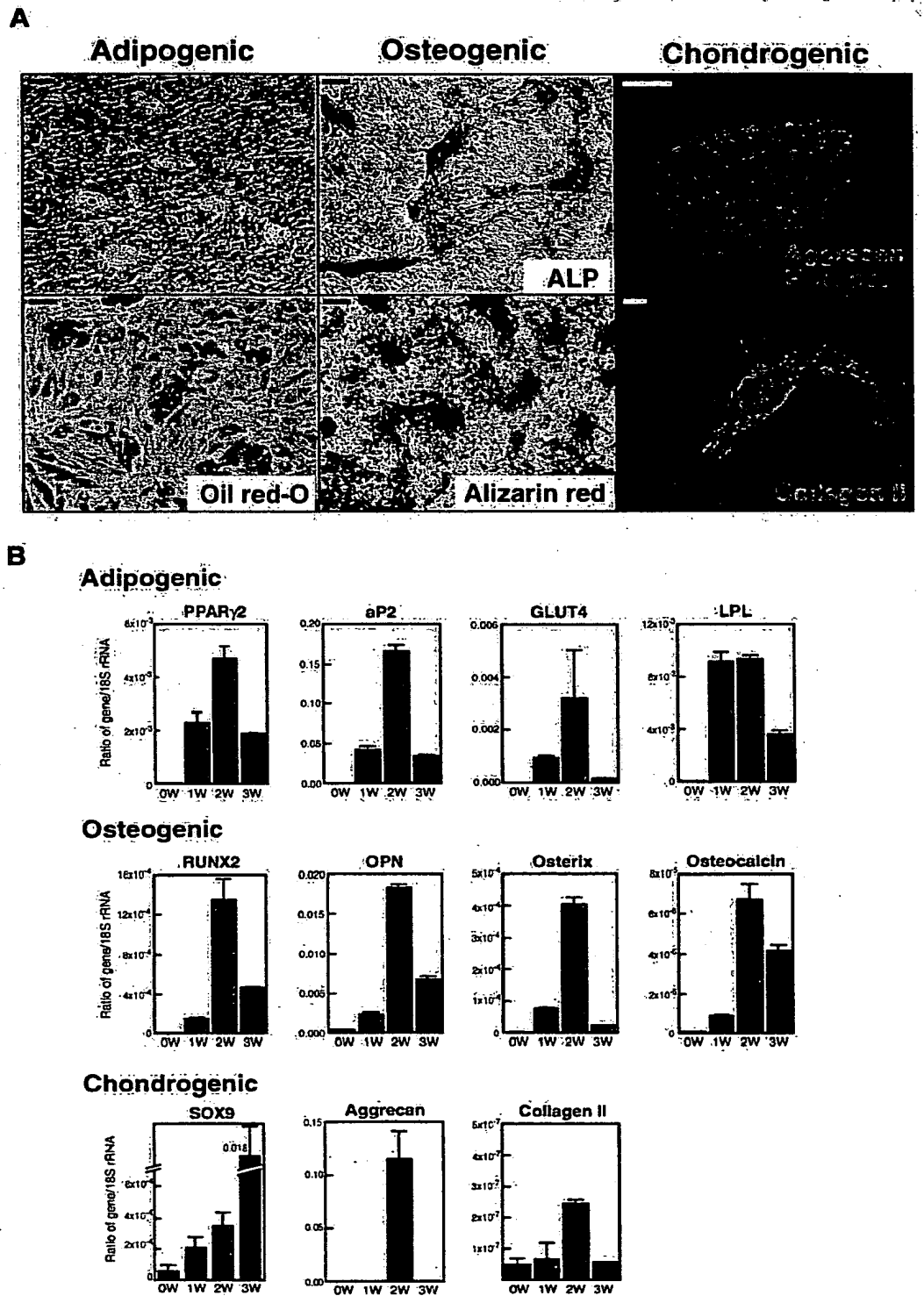


Fig. 7. Chondrogenic differentiation of DFAT cells. A,B: Confluent human DFAT cells were cultured for 3 weeks in chondrogenic medium. Cells were immunostained for collagen type II (A) and stained for alcian blue (B). Bar, 50 μm. C: HE staining of a section through a micromass pellet culture of DFAT cells after 3 weeks of chondrogenic differentiation. Bar, 50 μm. D: Expression of chondrogenic genes in DFAT cells was analyzed by real-time RT-PCR before (0 W) and after chondrogenic differentiation culture (1–3 W). Values are mean ± SD of triplicate dishes.

cycle and dedifferentiate into fibroblast-like DFAT cells when the cells are subjected to ceiling culture in the absence of any special factors, such as reversine. Recently, it was reported that the fibroblast-like cells in ceiling cultures are likely to be derived from precursor cells tightly attached to mature adipocytes (Miyazaki et al., 2005). In contrast, our data strongly suggest that the fibroblast-like cells are directly derived from mature adipocytes. We repeatedly observed that more than 97% of cells isolated from adipose tissues used for ceiling cultures were lipid-filled adipocytes with a single nucleus. Approximately 50% of the adherent cells showed BrdU incorporation and approximately 40% exhibited a fibroblast-like morphology by day 7 of culture (Fig. 2). These findings indicate that mature adipocytes gain the capacity for DNA synthesis and alter their morphology into a fibroblast-like appearance. Adipocytes with two-nuclei were rarely observed before ceiling culture, whereas they were frequently observed by day 3 of culture. These di-nuclear cells were always positive for BrdU in both nuclei (data not shown), indicating that the adipocyte itself entered S-phase and the nucleus had divided. In addition, time-lapse of fluorescence microscopy revealed that fibroblast-like cells were produced from lipid-filled adipocytes with a single nucleus via asymmetric division (see Supplementary video). Previous observations by other groups (Sugihara et al., 1986; Shigematsu et al., 1999; Zhang et al., 2000), in which mature adipocytes exhibit DNA synthesis during ceiling culture, also support the notion that the multipotent fibroblast-like cells are directly derived from mature adipocytes.

The cell surface antigen profile of DFAT cells was very similar to that of bone marrow MSCs, except DFAT cells expressed high levels of CD49d and little or no CD106. The expression profiles for the surface antigens we examined were essentially the same between DFAT cells and ASCs. Furthermore, the proliferation rate and differentiation capacity of DFAT cells are very similar to those of ASCs. These findings suggest that both DFAT cells and ASCs contain the same type of cells. On the other hand, flow cytometric analysis revealed that DFAT cells, in comparison to ASCs, are a highly homogeneous population of cells (Fig. 3B). Consistent with previously reported findings (Zuk et al., 2001; Yoshimura et al., 2006), we showed that ASCs at passage 1 contained high numbers of smooth muscle cells



COLOR

Fig. 8. Multilineage potential of clonally derived DFAT cells. A: Clonally expanded porcine DFAT cells were cultured for 3 weeks in appropriate induction media. Cells expanded from clone #2 were analyzed for osteogenic (ALP and alizarin red S), adipogenic (Oil red-O), and chondrogenic (aggrecan and collagen type II) capacity. Bar, 200 μm (osteogenic and adipogenic); 50 μm (chondrogenic). B: Expression of the indicated lineage-specific genes in cells expanded from clone #2 was analyzed by real-time RT-PCR before (0 W) and after the indicated differentiation culture (1–3 W). Values are mean ± SD of triplicate dishes.

(18.6%), endothelial cells (2.7%), and blood cells (12.8–13.3%). This result is convincing because DFAT cells originate from a fraction of highly homogeneous mature adipocytes, whereas ASCs are from SVF, which contains a variety of cell types.

We showed by real-time RT-PCR analysis that DFAT cells express mRNA for adipogenic markers such as PPARγ, C/EBPβ, and C/EBPδ, suggesting that DFAT cells retain the properties of adipose lineage-committed progenitor cells. To our surprise, DFAT cells also express mRNAs for the osteogenic marker Runx2 and chondrogenic marker SOX9 regardless of lineage-specific induction cultures. These data suggest that DFAT cells gain the properties of other mesenchymal lineage-committed progenitors. It needs to be clarified whether these genes are expressed together in single cells or expressed individually in different cells.

In the present study, we showed that single cell-derived clonal populations of DFAT cells differentiate into adipogenic, chondrogenic, and osteogenic cells in the presence of lineage-specific induction factors. To the best of our knowledge, this is the first report that dedifferentiated cells derived from mature adipocytes are capable of differentiation into multiple-mesenchymal lineages. This finding, coupled with the ability of DFATs to proliferate while retaining the capacity to differentiate, supports the hypothesis that DFAT cells have an adult stem cell character. Twenty-five percent of our clones had 3-lineage, 37.5% had 2-lineage, and 37.5% had 1-lineage differentiation potential. This result is consistent with those of previous reports (Pittenger et al., 1999; Muraglia et al., 2000; Zuk et al., 2002; Gronthos et al., 2003; Guilak et al., 2006) in which clonally-derived bone marrow MSCs and ASCs are heterogeneous in terms of their multilineage differentiation potential. DFAT cells may be composed of not only completely undifferentiated stem-like cells but also subpopulations at different states of differentiation.

Like bone marrow MSCs or ASCs, DFAT cells appear to be an attractive cell source for regenerative medicine because of their high proliferation rates for ex vivo expansion and multilineage differentiation capacity. In addition, DFAT cells have several properties that make them well suited for regenerative medicine. First, DFAT cells are quite homogeneous. Bone marrow MSC and ASC populations are obtained by expansion of a very small number of stem cells from highly heterogeneous cell populations, such as bone marrow cells and adipose tissue SVF, by exploiting the phenotype of plastic adherence. In these cultures, certain types of differentiated cells frequently contaminate the stem cell populations (Clark and Keating, 1995; Zuk et al., 2001). Therefore, several passages are usually needed to eliminate contamination (Digirolamo et al., 1999; Colter et al., 2000). In contrast, DFAT cells contain almost no the other cell types even at the first passage. This property of DFAT cells may lead to higher safety and efficacy for clinical cell therapies. Second, DFAT cells can be obtained from small amounts of adipose tissue. We reproducibly isolated $4–6 \times 10^6$ adipocytes from approximately 1 g of subcutaneous adipose tissue. We found that only 5×10^4 adipocytes are needed to obtain sufficient numbers of DFAT cells (3×10^6 at the primary culture) for significant expansion within a few passages. This suggests that adequate quantities of DFAT cells for tissue engineering can be prepared from less than 100 mg of adipose tissue. Third, DFAT cells can be obtained from donors regardless of their age. We successfully prepared DFAT cells from donors 4–81 years of age, although lower proliferative activity was observed in the cells from donors over 70 years of age. Multilineage differentiation potential of DFAT cells was confirmed in most donors we examined except for those of very high age such as 77 and 81 years. These observations suggest that DFAT cells can be used for autologous transplantation in patients of various of ages. Moreover, DFAT cells may be an attractive cell source for allogenic transplantation. Fat tissues can be easily collected from large numbers of healthy donors who undergo surgery or liposuction because these tissues are typically discarded after surgery. Therefore, DFAT cells collected from children or young adults can be stored in a cell bank and used for allogenic transplantation to other patients. Like MSCs and ASCs, DFAT cells express HLA-A, -B, and -C but not HLA-DR, suggesting high potential for allogenic transplantation. We found that human DFAT cells can form osteoid matrix in vivo, suggesting that DFAT cells can be used clinically when bone formation is required, for instance, in the repair of non-union fractures and large bone defects. Further studies are needed to evaluate the safety, functionality, and long-term viability of DFAT cell transplants.

In conclusion, our observations indicate that lipid-filled adipocytes can dedifferentiate into fibroblast-like DFAT cells that have the potential to transdifferentiate into lineages of mesenchymal tissue similar to MSCs. Although much research concerning a cell source for regenerating tissues and organs has focused on stem cells, we have focused that multipotent cells can be generated from fully differentiated cells, such as mature adipocytes. Because DFAT cells are easily isolated from a small amount of adipose tissue and are readily expanded with high purity, DFAT cells may be applicable to many tissue-engineering strategies and cell-based therapies.

Acknowledgments

We thank Dr. Yoshimitsu Nakano (Department of Plastic Surgery, Nihon University School of Medicine), Dr. Kenji Sasaki (Department of Plastic Surgery, Nihon University School of Medicine), Dr. Toshio Yoshida (Department of Urology, Nihon University School of Medicine), Dr. Satoshi Takahashi (Department of Urology, Nihon University School of Medicine), and Dr. Takeshi Kusafuka (Division of Pediatric Surgery, Department of Surgery, Nihon University School of Medicine) for human adipose tissues. We are also grateful to Dr. Kazumichi Kuroda for technical assistance with the time-lapse fluorescence microscopy. This work was supported by a Grant-in Aid for the High-Tech Research Center from the Ministry of Education, Science, Sports, and Culture of Japan to Nihon University (1550863), and by a Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan to promote open research for young academics and specialists to Nihon University. This work was also supported by a Nihon University Multidisciplinary Research Grant (2005, 2006).

Literature Cited

Andres V, Walsh K. 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J Cell Biol 132:657–666.

Barry[Q4] FP, Boynton RE, Haynesworth S, Murphy JM, Zaia J. 1999. The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). Biochem Biophys Res Commun 265:134–139.

Brockes JP, Kumar A. 2002. Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nat Rev Mol Cell Biol 3:566–574.

Chen S, Zhang Q, Wu X, Schultz PG, Ding S. 2004. Dedifferentiation of lineage-committed cells by a small molecule. J Am Chem Soc 126:410–411.

Clark BR, Keating A. 1995. Biology of bone marrow stroma. Ann NY Acad Sci 770:70–78.

Colter DC, Class R, DiGirolamo CM, Prockop DJ. 2000. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci USA 97:3213–3218.

Del Rio-Tsonis K, Tsonis PA. 2003. Eye regeneration at the molecular age. Dev Dyn 226:211–224.

Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. 1999. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107:275–281.

Gronthos S, Zannettino AC, Hay SJ, Shi S, Graves SE, Kortesidis A, Simmons PJ. 2003. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci 116:1827–1835.

Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, Gimble JM. 2006. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. J Cell Physiol 206:229–237.

Justesen J, Pedersen SB, Stenderup K, Kassem M. 2004. Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo. Tissue Eng 10:381–391.

Matsumoto T, Turesson I, Book M, Gerwins P, Claesson-Welsh L. 2002. p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. J Cell Biol 156:149–160.
McGann CJ, Odelberg SJ, Keating MT. 2001. Mammalian myotube dedifferentiation induced by newt regeneration extract. Proc Natl Acad Sci USA 98:13699–13704.
McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, Kloster A, Di Halvorsen Y, Ting JP, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. 2006. The immunogenicity of human adipose-derived cells: Temporal changes in vitro. Stem Cells 24:1246–1253.
Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. 2006. Immunophenotype of human adipose-derived cells: Temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 24:376–385.
Miyazaki T, Kitagawa Y, Toriyama K, Kobori M, Torii S. 2005. Isolation of two human fibroblastic cell populations with multiple but distinct potential of mesenchymal differentiation by ceiling culture of mature fat cells from subcutaneous adipose tissue. Differentiation 73:69–78.
Muraglia A, Cancedda R, Quarto R. 2000. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113:1161–1166.
Pettersson P, Van R, Karlsson M, Bjorntorp P. 1985. Adipocyte precursor cells in obese and nonobese humans. Metabolism 34:808–812.
Pittenger MF, Martin BJ. 2004. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 95:9–20.
Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.
Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L, Casteilla L. 2004. Plasticity of human adipose lineage cells toward endothelial cells: Physiological and therapeutic perspectives. Circulation 109:656–663.
Schaffler A, Buchler C. 2007. Concise review: Adipose tissue-derived stromal cells–basic and clinical implications for novel cell-based therapies. Stem Cells 25:818–827.
Shigematsu M, Watanabe H, Sugihara H. 1999. Proliferation and differentiation of unilocular fat cells in the bone marrow. Cell Struct Funct 24:89–100.
Strem BM, Hicok KC, Zhu M, Wulur I, Alfonso Z, Schreiber RE, Fraser JK, Hedrick MH. 2005. Multipotential differentiation of adipose tissue-derived stem cells. Keio J Med 54:132–141.
Sugihara H, Yonemitsu N, Miyabara S, Yun K. 1986. Primary cultures of unilocular fat cells: Characteristics of growth in vitro and changes in differentiation properties. Differentiation 31:42–49.
Sugihara H, Yonemitsu N, Miyabara S, Toda S. 1987. Proliferation of unilocular fat cells in the primary culture. J Lipid Res 28:1038–1045.
Sugihara H, Funatsumaru S, Yonemitsu N, Miyabara S, Toda S, Hikichi Y. 1989. A simple culture method of fat cells from mature fat tissue fragments. J Lipid Res 30:1987–1995.
Tsonis PA. 2000. Regeneration in vertebrates. Dev Biol 221:273–284.
Walsh K, Perlman H. 1997. Cell cycle exit upon myogenic differentiation. Curr Opin Genet Dev 7:597–602.
Yagi K, Kondo D, Okazaki Y, Kano K. 2004. A novel preadipocyte cell line established from mouse adult mature adipocytes. Biochem Biophys Res Commun 321:967–974.
Yamasaki M, Emoto H, Konishi M, Mikami T, Ohuchi H, Nakao K, Itoh N. 1999. FGF-10 is a growth factor for preadipocytes in white adipose tissue. Biochem Biophys Res Commun 258:109–112.
Yoshimura K, Shigeura T, Matsumoto D, Sato T, Takaki Y, Aiba-Kojima E, Sato K, Inoue K, Nagase T, Koshima I, Gonda K. 2006. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol 208:64–76.
Zhang HH, Kumar S, Barnett AH, Eggo MC. 2000. Ceiling culture of mature human adipocytes: Use in studies of adipocyte functions. J Endocrinol 164:119–128.
Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. 2001. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 7:211–228.
Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295.

Q1: Author: Please check the grant sponsors.

Q2: Author: Please provide complete location.

Q3: Author: Please provide the column head for the second column.

Q4: Author: Please provide the citation in the text.

WILEY
Publishers Since 1807

COLOR REPRODUCTION IN YOUR ARTICLE

These proofs have been typeset using figure files transmitted to production when this article was accepted for publication. Please review all figures and note your approval with your submitted proof corrections. You may contact the journal production team at **JCPprod@wiley.com** if you wish to discuss specific concerns.

Because of the high cost of color printing, we can only print figures in color if authors cover the expense. If you have submitted color figures, please indicate your consent to cover the cost on the table listed below by marking the box corresponding to the approved cost on the table. The rate for this journal is $500 USD per printed page of color, regardless on the number of figures appearing on that page.

Please note, all color images will be reproduced online in Wiley *InterScience* at no charge, whether or not you opt for color printing.

You will be invoiced for color charges once the article has been published in print.

Failure to return this form with your article proofs may delay the publication of your article.

JOURNAL **JOURNAL OF CELLULAR PHYSIOLOGY** MS. NO. _____ NO. COLOR PAGES _____

MANUSCRIPT TITLE _____

AUTHOR(S) _____

No. Color Pages	Color Charge	No. Color Pages	Color Charge	No. Color Pages	Color Charge
☐ 1	$500	☐ 5	$2500	☐ 9	$4500
☐ 2	$1000	☐ 6	$3000	☐ 10	$5000
☐ 3	$1500	☐ 7	$3500	☐ 11	$5500
☐ 4	$2000	☐ 8	$4000	☐ 12	$6000
Contact JCPprod@wiley.com for a quote if you have more than 12 pages of color					

☐ Please print my figures color

☐ Please print my figures in black and white

☐ Please print the following figures in color _____

and convert these figures to black and white _____

Approved by _____

Billing Address _____ E-mail _____

_____ Telephone _____

_____ Fax _____

C1

WILEY

Publishers Since 1807

REPRINT BILLING DEPARTMENT • 111 RIVER STREET • HOBOKEN, NJ 07030
FAX: (201) 748-7670
E-MAIL: reprints@wiley.com

PREPUBLICATION REPRINT ORDER FORM

Please complete this form even if you are not ordering reprints. This form **MUST** be returned with your corrected proofs and original manuscript. Your reprints will be shipped approximately 4 weeks after publication. Reprints ordered after printing will be substantially more expensive.

JOURNAL *JOURNAL OF CELLULAR PHYSIOLOGY* VOLUME _____ ISSUE _____
TITLE OF MANUSCRIPT _____
MS. NO. _____ NO. OF PAGES _____ AUTHOR(S) _____

No. of Pages	100 Reprints	200 Reprints	300 Reprints	400 Reprints	500 Reprints
	$	$	$	$	$
1-4	336	501	694	890	1052
5-8	469	703	987	1251	1477
9-12	594	923	1234	1565	1850
13-16	714	1156	1527	1901	2273
17-20	794	1340	1775	2212	2648
21-24	911	1529	2031	2536	3037
25-28	1004	1707	2267	2828	3388
29-32	1108	1894	2515	3135	3755
33-36	1219	2092	2773	3456	4143
37-40	1329	2290	3033	3776	4528

**REPRINTS ARE ONLY AVAILABLE IN LOTS OF 100. IF YOU WISH TO ORDER MORE THAN 500 REPRINTS, PLEASE CONTACT OUR REPRINTS DEPARTMENT AT (201) 748-6353 FOR A PRICE QUOTE.

☐ Please send me _____ reprints of the above article at $ _____

Please add appropriate State and Local Tax for United States orders only. (Tax Exempt No._____) $ _____

Please add 5% Postage and Handling $ _____

TOTAL AMOUNT OF ORDER** $ _____

***International orders must be paid in currency and drawn on a U.S. bank*

Please check one: ☐ Check enclosed ☐ Bill me ☐ Credit Card
If credit card order, charge to: ☐ American Express ☐ Visa ☐ MasterCard

Credit Card No _____ Signature _____ Exp. Date _____

BILL TO:
Name _____
Institution _____
Address _____
_____
Purchase Order No. _____

SHIP TO: (Please, no P.O. Box numbers)
Name _____
Institution _____
Address _____
_____
Phone _____ Fax _____
E-mail _____

WILEY
Publishers Since 1807

111 River Street
Hoboken, NJ 07030

COPYRIGHT TRANSFER AGREEMENT

Date:

To:

Production/Contribution ID#_____ Publisher/Editorial office use only

Re: Manuscript entitled_____
_____ (the "Contribution")
for publication in ***JOURNAL OF CELLULAR PHYSIOLOGY***_____ (the "Journal")
published by Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. ("Wiley").

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the publishing process and enable Wiley to disseminate your work to the fullest extent, we need to have this Copyright Transfer Agreement signed and returned to us as soon as possible. If the Contribution is not accepted for publication this Agreement shall be null and void.

A. COPYRIGHT

1. The Contributor assigns to Wiley, during the full term of copyright and any extensions or renewals of that term, all copyright in and to the Contribution, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution and the material contained therein in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

2. Reproduction, posting, transmission or other distribution or use of the Contribution or any material contained therein, in any medium as permitted hereunder, requires a citation to the Journal and an appropriate credit to Wiley as Publisher, suitable in form and content as follows: (Title of Article, Author, Journal Title and Volume/Issue Copyright © [year] Wiley-Liss, Inc. or copyright owner as specified in the Journal.)

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's Employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution, and the right to make oral presentations of material from the Contribution.

C. OTHER RIGHTS OF CONTRIBUTOR

Wiley grants back to the Contributor the following:

1. The right to share with colleagues print or electronic "preprints" of the unpublished Contribution, in form and content as accepted by Wiley for publication in the Journal. Such preprints may be posted as electronic files on the Contributor's own website for personal or professional use, or on the Contributor's internal university or corporate networks/intranet, or secure external website at the Contributor's institution, but not for commercial sale or for any systematic external distribution by a third party (e.g., a listserve or database connected to a public access server). Prior to publication, the Contributor must include the following notice on the preprint: "This is a preprint of an article accepted for publication in [Journal title] © copyright (year) (copyright owner as specified in the Journal)". After publication of the Contribution by Wiley, the preprint notice should be amended to read as follows: "This is a preprint of an article published in [include the complete citation information for the final version of the Contribution as published in the print edition of the Journal]", and should provide an electronic link to the Journal's WWW site, located at the following Wiley URL: http://www.interscience.Wiley.com/. The Contributor agrees not to update the preprint or replace it with the published version of the Contribution.

2. The right, without charge, to photocopy or to transmit online or to download, print out and distribute to a colleague a copy of the published Contribution in whole or in part, for the Contributor's personal or professional use, for the advancement of scholarly or scientific research or study, or for corporate informational purposes in accordance with Paragraph D.2 below.

3. The right to republish, without charge, in print format, all or part of the material from the published Contribution in a book written or edited by the Contributor.

4. The right to use selected figures and tables, and selected text (up to 250 words, exclusive of the abstract) from the Contribution, for the Contributor's own teaching purposes, or for incorporation within another work by the Contributor that is made part of an edited work published (in print or electronic format) by a third party, or for presentation in electronic format on an internal computer network or external website of the Contributor or the Contributor's employer.

5. The right to include the Contribution in a compilation for classroom use (course packs) to be distributed to students at the Contributor's institution free of charge or to be stored in electronic format in datarooms for access by students at the Contributor's institution as part of their course work (sometimes called "electronic reserve rooms") and for in-house training programs at the Contributor's employer.

D. CONTRIBUTIONS OWNED BY EMPLOYER

1. If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/employer which must sign this Agreement (in addition to the Contributor's signature), in the space provided below. In such case, the company/employer hereby assigns to Wiley, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

2. In addition to the rights specified as retained in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, Wiley hereby grants back, without charge, to such company/employer, its subsidiaries and divisions, the right to make copies of and distribute the published Contribution internally in print format or electronically on the Company's internal network. Upon payment of the Publisher's reprint fee, the institution may distribute (but not resell) print copies of the published Contribution externally. Although copies so made shall not be available for individual re-sale, they may be included by the company/employer as part of an information package included with software or other products offered for sale or license. Posting of the published Contribution by the institution on a public access website may only be done with Wiley's written permission, and payment of any applicable fee(s).

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government Employees: see note at end).

F. COPYRIGHT NOTICE

The Contributor and the company/employer agree that any and all copies of the Contribution or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal as published by Wiley.

G. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that the Contribution is the Contributor's original work. If the Contribution was prepared jointly, the Contributor agrees to inform the co-Contributors of the terms of this Agreement and to obtain their signature to this Agreement or their written permission to sign on their behalf. The Contribution is submitted only to this Journal and has not been published before, except for "preprints" as permitted above. (If excerpts from copyrighted works owned by third parties are included, the Contributor will obtain written permission from the copyright owners for all uses as set forth in Wiley's permissions form or in the Journal's Instructions for Contributors, and show credit to the sources in the Contribution.) The Contributor also warrants that the Contribution contains no libelous or unlawful statements, does not infringe on the rights or privacy of others, or contain material or instructions that might cause harm or injury.

CHECK ONE:

[____]Contributor-owned work

Contributor's signature _____ Date _____

Type or print name and title _____

Co-contributor's signature _____ Date _____

Type or print name and title _____

ATTACH ADDITIONAL SIGNATURE PAGE AS NECESSARY

[____]Company/Institution-owned work (made-for-hire in the course of employment)

Company or Institution (Employer-for-Hire) _____ Date _____

Authorized signature of Employer _____ Date _____

[____]**U.S. Government work**

Note to U.S. Government Employees

A Contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. Government publication is called a "U.S. Government work," and is in the public domain in the United States. In such case, the employee may cross out Paragraph A.1 but must sign and return this Agreement. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. Government publication, it is not a U.S. Government work.

[____]**U.K. Government work (Crown Copyright)**

Note to U.K. Government Employees

The rights in a Contribution prepared by an employee of a U.K. government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown. In such case, the Publisher will forward the relevant form to the Employee for signature.